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## Membrane-anchored aspartyl protease with Alzheimer's disease $\beta$ -secretase activity

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Mutations in the gene encoding the amyloid protein precursor (APP) cause autosomal dominant Alzheimer's disease<sup>1–3</sup>. Cleavage of APP by unidentified proteases, referred to as  $\beta$ - and  $\gamma$ -secretases<sup>4–7</sup>, generates the amyloid  $\beta$ -peptide, the main component of the amyloid plaques found in Alzheimer's disease patients<sup>8</sup>. The disease-causing mutations flank the protease cleavage sites in APP and facilitate its cleavage. Here we identify a new membrane-bound aspartyl protease (Asp2) with  $\beta$ -secretase activity. The Asp2 gene is expressed widely in brain and other tissues. Decreasing the expression of Asp2 in cells reduces amyloid  $\beta$ -peptide production and blocks the accumulation of the carboxy-terminal APP fragment that is created by  $\beta$ -secretase cleavage. Solubilized Asp2 protein cleaves a synthetic APP peptide substrate at the  $\beta$ -secretase site, and the rate of cleavage is increased tenfold by a mutation associated with early-onset Alzheimer's disease in Sweden<sup>3</sup>. Thus, Asp2 is a new protein target for drugs that are designed to block the production of amyloid  $\beta$ -peptide and the consequent formation of amyloid plaque in Alzheimer's disease.

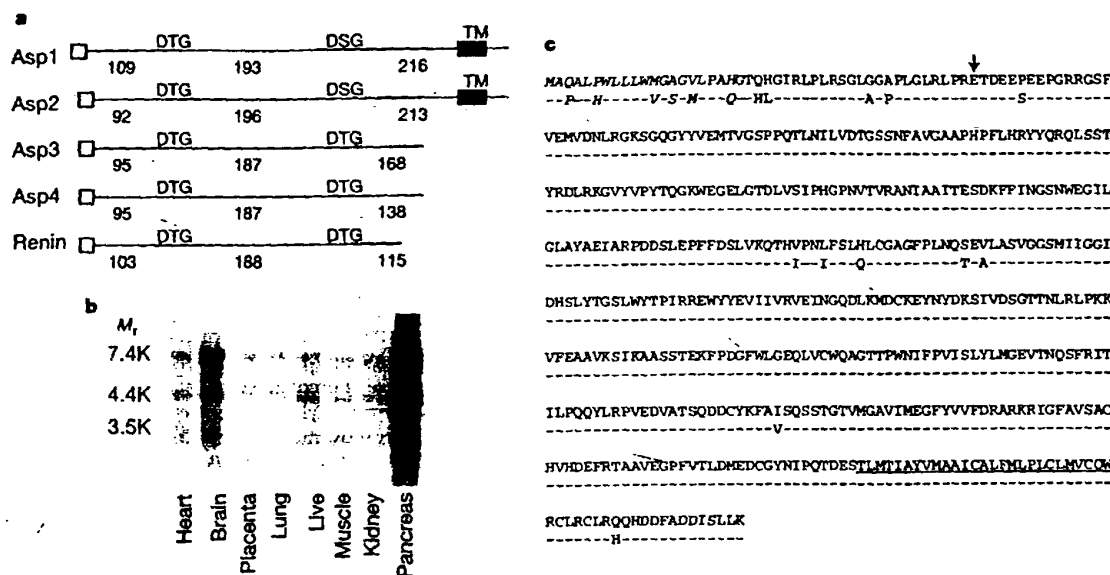
Visual inspection suggests that the  $\beta$ - and  $\gamma$ -secretase cleavage sites in APP might be substrates for cleavage by aspartyl proteases,

and indeed, cathepsin D cleaves synthetic  $\beta$ -secretase substrates<sup>9</sup>. This cleavage is facilitated by the KM  $\rightarrow$  NL mutation, referred to as the 'Swedish' mutation, found in patients with early-onset Alzheimer's disease<sup>10</sup>; however, APP processing to amyloid  $\beta$  (A $\beta$ ) peptides occurs normally in hippocampal neurons cultured from cathepsin-D-null mice<sup>11</sup>. Nevertheless, it seemed plausible that the APP  $\beta$ - or  $\gamma$ -secretases could be as yet uncharacterized aspartyl proteases; therefore, we searched for new human enzymes of this mechanistic set. Sequencing of the *Caenorhabditis elegans* genome was nearing completion, which offered the possibility of enumerating the complete set of aspartyl proteases encoded in a simple metazoan genome, and using these as a bridge to human sequence databases.

Simple AWK scripts scanning for the D(S/T)G active-site motif, PROSITE and hidden Markov models were used to search the WormPep database of predicted *C. elegans* proteins. This revealed at least 10 candidate aspartyl proteases. Seven of these ten were found on a single chromosome, chromosome V (F21F8.3, F21F8.4, F21F8.7, Y39B6B.G, Y39B6B.J, Y39B6B.H and T18H9.2), and three each of these were found in the same cosmid clones (F21F8 and Y39B6B), suggesting that they represent a recently evolved family of proteins that arose by ancestral gene duplication. Other homologous predicted genes were found in the same cluster (F21F8.2, F21F8.6 and Y39B6B.I); however, these contain only a single DTG or DSG motif. Additional predicted aspartyl protease genes were found on chromosomes IV (C11D2.2) and X (R12H7.2 and H22K11.1). Searches of vertebrate expressed sequence tag (EST) databases with the 10 *C. elegans* sequences identified 7 known and 4 new candidate aspartyl proteases. The new human sequences were numbered in order of their discovery (Asp1–4). R12H7.2 and H22K11.1 appear to be *C. elegans* homologues of cathepsin D. Most of the chromosome V aspartyl proteases had no clear vertebrate orthologues; however, one of these (T18H9.2) bridged to two unusual sequences (Asp1 and Asp2) which contained the less common DSG motif in the second active site. In turn, C11D2.2 identified two additional sequences (Asp3 and Asp4) which have since been reported in the literature as napsins A and B<sup>12</sup>.

The two predicted aspartyl protease sequences identified by T18H9.2 were of greatest interest. Completion of their sequences by a combination of EST sequencing, 5' rapid amplification of complementary DNA ends by the polymerase chain reaction, and library screening showed that both Asp1 and Asp2 had an unusual C-terminal extension containing a single predicted transmembrane domain (Fig. 1). Asp1 maps to human chromosome 21q22 within the Down's syndrome critical region, and Asp2 to chromosome 11q23–24. Northern hybridization to human tissue blots showed widespread expression of both Asp1 and Asp2. Both are expressed at the highest levels in pancreas. Asp2 is also expressed at high levels in brain, whereas Asp1 is expressed in brain at somewhat lower levels. *In situ* hybridization showed expression of Asp2 primarily in acinar cells of the exocrine pancreas, whereas faint hybridization was seen over neurons in hippocampus; however, we identified two Asp2 EST in a human astrocyte cDNA library indicating that Asp2 may be expressed in both neurons and glial cells. Transcripts for both Asp1 and Asp2 were expressed in human embryonic kidney 293 cells, human IMR-32 neuroblastoma cells and mouse Neuro-2a neuroblastoma cells, three commonly used cellular models of APP processing.

We used a panel of antisense oligomers to test the involvement of each of the four predicted aspartyl proteases in APP processing by a stable clone of HEK293 cells that had been engineered to process APP to A $\beta$  peptides at high levels. These cells were transformed with a modified human APP695 cDNA containing the Swedish KM  $\rightarrow$  NL mutation to which two lysine residues had been added to the C terminus (HEK/APP-Sw-KK cells). The KK motif greatly increases the processing and release of A $\beta$  peptides but does not influence the ratio of A $\beta$ 42/(A $\beta$ 42 + A $\beta$ 40), nor alter the effect of



**Figure 1** Asp2 functional domains, tissue distribution and amino-acid sequence. **a**, Alignment of the predicted primary structures of Asp1–Asp4 with human renin. Asp1 and Asp2 (51% amino-acid identity) have a DSG motif in the second predicted active site and possess an unusual C-terminal extension containing a single predicted transmembrane domain (TM). The predicted signal peptide for secretion (open box) and the number of residues in each domain is indicated. **b**, Tissue distribution of human Asp2.

the KM → NL or V717F mutations. These mutations increase total Aβ processing<sup>4,5</sup> or selectively increase the production of Aβ42<sup>6</sup>, respectively. Thus, the HEK/APP-Sw-KK cells provide a sensitized background on which to screen for inhibition of Aβ processing.

Transfection of HEK/APP-Sw-KK cells with the panel of 16 antisense oligomers (four each targeting Asp1–Asp4) showed that only those oligomers targeting Asp2 considerably decreased the release of Aβ peptides into the medium. Two of the Asp2 antisense oligomers were chosen for resynthesis, as well as for synthesis of two additional oligomers containing the reverse sequence for use as controls. Their effects on transfected HEK/APP-Sw-KK cells are shown in Fig. 2. Both of the antisense oligomers targeting the human Asp2 transcript reduced Asp2 message levels, whereas the control reverse oligomers did not. Both also reduced the release of Aβ peptides into the culture medium. The inhibition of Aβ release ranged from 50% to 80% in many separate experiments and probably was dependent upon transfection efficiency. The antisense oligomers reduced the production of both Aβ40 and Aβ42 by roughly the same amount. The reduction of Aβ peptide production also was confirmed by immunoprecipitation and western blotting. This indicates that Asp2 may be involved directly or indirectly in the production of Aβ peptides and their release from HEK293 cells.

Because HEK293 cells derive from kidney, we extended the experiment to human IMR-32 neuroblastoma cells, which express all three APP isoforms<sup>13</sup> and which release Aβ peptides into conditioned medium at measurable concentrations<sup>14</sup>, and obtained essentially identical results. The Asp2-1A and Asp2-2A antisense oligomers reduced Asp2 messenger RNA by 75% and 39%, respectively (quantitated using a TaqMan probe), whereas the reverse control oligomers had no effect. Correspondingly, release of Aβ40 and Aβ42 was reduced by 49 ± 2% and 42 ± 14% from cells treated with Asp2-1A, and by 43 ± 3 and 44 ± 18 with Asp2-2A (*P* < 0.001). Again, the reverse control oligomers had no effect.

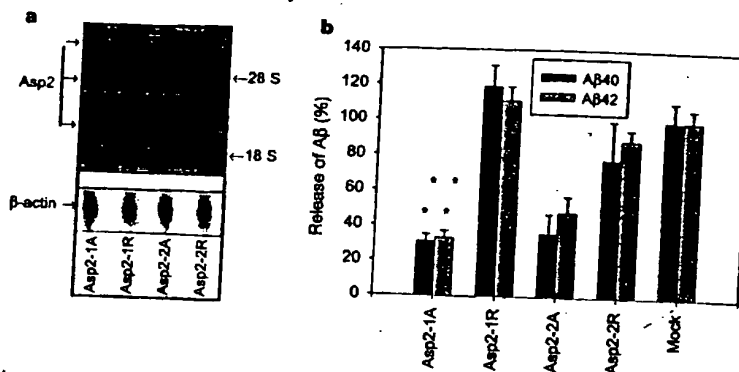
Similar results were obtained in a murine system. Molecular cloning of the mouse Asp2 cDNA revealed a remarkable 98% amino-acid identity to human (Fig. 1c) and complete nucleotide

mRNA expression as shown by northern hybridization. The relative molecular mass (*M*<sub>r</sub>) of each transcript is indicated. **c**, Clustal W sequence alignment of Asp2 from human (top line) and mouse (second line). The signal peptide is indicated in italics, the predicted transmembrane domain is underlined, and the active-site sequences are in bold. Arrow indicates the N terminus of purified recombinant Asp2 expressed in CHO cells.

identity at the sites targeted by the Asp2-1A and Asp2-2A antisense oligomers. In mouse Neuro-2A cells engineered to express APP-Sw-KK, the Asp2-1A antisense oligomer reduced the release of Aβ40 and Aβ42 by 70 ± 7% and 67 ± 2%, whereas a reduction of 61 ± 12% was seen for the release of both Aβ40 and Aβ42 from cells treated with Asp2-2A (*P* < 0.001). The reverse control oligomers had no effect. Thus, the three antisense experiments with HEK293, IMR-32 and Neuro-2a cells indicate that Asp2 is directly or indirectly involved in Aβ processing in both somatic and neural cell lines.

Treatment of HEK293/APP-Sw-KK cells with the Asp2 antisense oligomers had little effect on the release of total soluble APP (sAPP) from cells although they did appear to alter the ratio of sAPP isoforms released by either α- or β-secretase cleavage (Fig. 3). These cleavages generate species of soluble APP (sAPPα and sAPPβ, respectively), which contain different C termini which can be distinguished by the 6E10 monoclonal antibody that recognizes Aβ residues 1–16. As shown in Fig. 3b, no change in the release of total sAPP is shown on western blots developed with the 22C11 monoclonal antibody that reacts with an amino-terminal epitope of APP, whereas development with 6E10 shows an increase in sAPPα release from cells treated with either of the Asp2 antisense oligomers. An enzyme-linked immunosorbent assay (EIA) specific for sAPPα showed that release increased at least twofold, from 2.8 μg ml<sup>-1</sup> to 6.7 μg ml<sup>-1</sup> (*P* < 0.005), in cells transfected with the Asp2-2 antisense oligomer.

Cleavage of APP at either the β- or α-secretase sites also leaves C-terminal fragments containing the APP transmembrane domain and cytoplasmic tail. These contain 99 and 83 amino acids (CTF99 and CTF83), respectively (Fig. 3a). Indeed, in HEK293/APP-Sw-KK cells treated with Asp2 antisense oligomers, the amount of the CTF99 β-secretase product is reduced (Fig. 3c). Correspondingly, co-transfection of HEK293 cells or Neuro-2A cells with human Asp2 and APP-KK increases the production of CTF99 in comparison with cells co-transfected with APP-KK and empty vector DNA. Production of CTF99 is increased still further in cells co-transfected



**Figure 2** Asp2 antisense oligomers decrease amyloid  $\beta$ -peptide processing. **a**, Asp2 antisense oligomers (Asp2-1A and Asp2-2A) targeting two different sites on the Asp2 transcript specifically reduce Asp2 mRNA in transfected HEK/APP-Sw-KK cells as determined by northern hybridization, whereas control oligomers (Asp2-1R and Asp2-2R) with the reverse sequence lacked this effect. Arrows indicate the three Asp2 transcripts

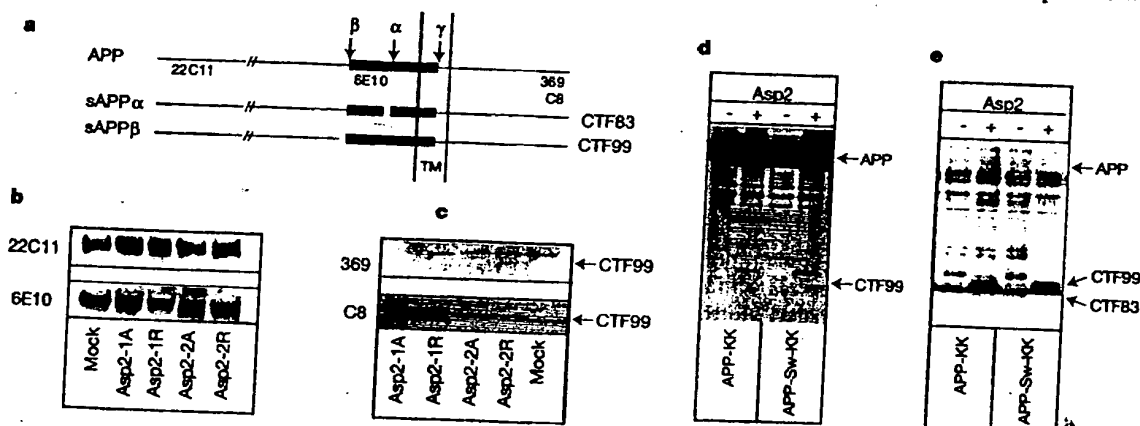
with constructs expressing Asp2 and APP-Sw-KK. Thus, Asp2 appears specifically to facilitate  $\beta$ -secretase cleavage of APP and this effect is enhanced by the Swedish KM  $\rightarrow$  NL mutation. This is in contrast to the effects of presenilin-1 gene disruption, which specifically increases the accumulation of CTF99 in cultured mouse neurons because of inhibition of  $\gamma$ -secretase cleavage<sup>15,16</sup>.

Effects of Asp2 on the production of A $\beta$  peptides from endogenously expressed APP isoforms were assessed in HEK293 cells transfected with a construct expressing Asp2 or with the empty vector after selection of transformants with the antibiotic G418. A $\beta$ 40 production was increased in cells transformed with the Asp2 construct in comparison with those transformed with the empty vector DNA, the concentrations in conditioned medium were  $424 \pm 45 \text{ pg ml}^{-1}$  and  $113 \pm 58 \text{ pg ml}^{-1}$ , respectively ( $P < 0.001$ ). A $\beta$ 42 release was below the limit of detection by the EIA, whereas the release of sAPP $\alpha$  was unaffected,  $112 \pm 8 \text{ ng ml}^{-1}$  versus  $111 \pm 40 \text{ ng ml}^{-1}$ . These results provide further support for the hypothesis that Asp2 functions in the processing and release of A $\beta$  from endogenously expressed APP.

present in HEK293 cells. No change was seen in  $\beta$ -actin mRNA in transfected cells. **b**, Release of A $\beta$ 40 and A $\beta$ 42 from HEK/APP-Sw-KK cells, as measured by EIA, was reduced specifically by the Asp2 antisense oligomers (asterisks,  $P < 0.001$ ). Antisense oligomers were transfected in quadruplicate cultures. Release was normalized against values for Mock-transfected cells treated with oligofectin-G only.

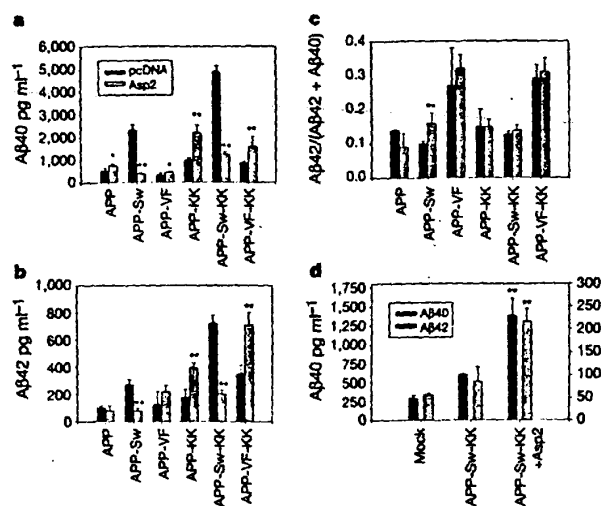
To determine the effects of Asp2 on the production of A $\beta$  peptides from mutant APP, we transfected the two pools of cells with a panel of APP constructs. This showed that co-expression of Asp2 with APP or APP-VF increased A $\beta$ 40 release from cells by 44% and 36%, respectively ( $P < 0.05$ ), and that this effect was magnified by addition of the KK motif (126% and 186%, respectively,  $P < 0.001$ ), Fig. 4a. Consistent with other reports<sup>6</sup>, the V717F mutation increased the production of A $\beta$ 42 relative to total A $\beta$  peptides, which was further increased to 175% by co-expression of Asp2 ( $P < 0.001$ ), but there was no change in the relative ratio, Fig. 4b, c. Thus, Asp2 had little effect on the choice of  $\gamma$ -secretase cleavage sites in transfected HEK293 cells.

Co-expression of Asp2 in HEK293 cells with constructs containing the Swedish KM  $\rightarrow$  NL mutation did not have the same effect on A $\beta$  production. Consistent with other reports<sup>45</sup>, the Swedish mutation increased the production of both A $\beta$ 40 and A $\beta$ 42 with no change in their relative ratio (Fig. 4). However, when Asp2 was overexpressed in HEK293 cells, co-expression of APP-Sw caused a decrease in A $\beta$ 40 and A $\beta$ 42 release in comparison with control



**Figure 3** Asp2 increases production of the APP C-terminal  $\beta$ -secretase product. **a**, Illustration of the  $\beta$ -,  $\alpha$ - and  $\gamma$ -secretase cleavage sites in APP and location of the 22C11, 6E10, 369 and C8 epitopes. The location of the A $\beta$  peptide within APP is indicated (box). Processing at the  $\alpha$ -secretase site cleaves the mid-region of the A $\beta$  sequence and liberates the sAPP $\alpha$  ectodomain containing the 6E10 epitope, whereas the consequent 83-amino-acid C-terminal fragment (CTF83) retains the APP transmembrane domain (TM). Processing at the  $\beta$ -secretase site releases the sAPP $\beta$  ectodomain and creates a 99-amino-acid C-terminal fragment (CTF99) containing the 6E10 epitope. **b**, Equal amounts of conditioned culture supernatants from HEK/APP-Sw-KK cells were analysed on western blots developed with the 22C11 and 6E10 antibodies. Cultures were

treated with the oligomers or transfection reagent as indicated. **c**, Equal amounts of protein from lysates of HEK/APP-Sw-KK cells were immunoprecipitated with either the 369 or C8 antibody as indicated, and analysed on western blots developed with 6E10 to identify CTF99. Cultures were treated with the oligomers or transfection reagent as indicated. **d**, Neuro-2A cells were co-transfected with either APP-KK or APP-Sw-KK, with or without Asp2 as indicated. Equal amounts of protein from cell lysates were analysed by western blot developed with 6E10 to detect APP and CTF99 (arrows). **e**, HEK293 were co-transfected with either APP-KK or APP-Sw-KK, with or without Asp2 as indicated. Equal amounts of protein from cell lysates were analysed by western blot developed with 369 antibody to detect APP, CTF99 and CTF83 (arrows).



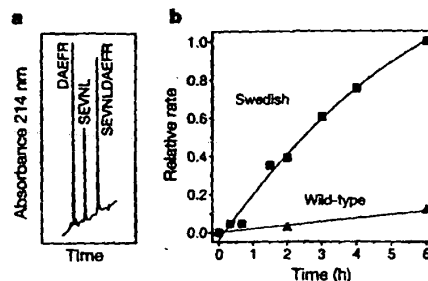
**Figure 4** Asp2 increases Aβ peptide release. **a**, Production of Aβ40 from HEK293 cells transfected with Asp2 or empty vector DNA (pcDNA) subsequently transfected with the indicated APP constructs. **b**, Production of Aβ42 as in **a**. **c**, Ratio of Aβ42/(Aβ42+Aβ40) produced as in **a**. The APP constructs were transfected in quadruplicate cultures (asterisk,  $P < 0.05$ ; double asterisk,  $P < 0.001$ ). **d**, Production of Aβ40 and Aβ42 by IMR-32 cells co-transfected with APP-Sw-KK and either Asp2 or empty vector DNA.

cells transformed with the empty vector. This effect is cell-line-dependent as co-transfection of IMR-32 cells with vectors expressing Asp2 and APP-Sw-KK results in more than a twofold increase in the production of both Aβ40 and Aβ42 in comparison with cells co-transfected with the APP-Sw-KK construct and empty vector DNA (Fig. 4d). Differences in the level of Asp2 expression or its localization within HEK293 or IMR-32 cells may account for this difference.

We obtained direct evidence that Asp2 possesses β-secretase activity using biochemical studies that measured purified Asp2 proteolytic activity against synthetic APP peptide substrates. Native, full-length Asp2 was expressed in Chinese hamster ovary (CHO) cells. Its behaviour on cell fractionation, detergent solubilization and purification by sequential chromatography was consistent with that of an integral membrane protein. Sequence analysis of the purified recombinant protein indicated a major N-terminal sequence beginning with a glutamic acid (arrow in Fig. 1c); however, at present, it is unclear whether this is the N terminus of mature Asp2.

Two peptides were designed for assaying β-secretase activity. The first contained the wild-type APP β-secretase site, whereas the second contained the Swedish KM → NL modification of the β-secretase cleavage site. Maximal activity was seen with the Swedish β-secretase peptide. As expected for an aspartyl protease, proteolytic activity was sensitive to pH with maximal hydrolysis seen at pH 5.0. Amino-terminal sequencing of the two cleavage products verified that cleavage occurred at the sequence NL ↓ DA (Fig. 5a). The rate of cleavage was reduced tenfold in the corresponding wild-type APP peptide (Fig. 5b). Proteolytic activity was insensitive to 8 μM pepstatin or a mixture of 10 μM leupeptin, 10 μM E-64 and 5 mM EDTA, inhibitors of cathepsin D (and other aspartyl proteases), serine proteases, cysteinyl proteases, and metalloproteases, respectively. 'Mock' preparations of unmodified CHO cell membranes did not contain substantial Asp2-like activity. Thus, Asp2 acts directly in cell-free assays to cleave synthetic APP peptides at the β-secretase site, and the rate of cleavage is greatly increased by the Swedish KM → NL mutation associated with Alzheimer's disease.

Our experiments associated a new human aspartyl protease with the processing of APP at the β-secretase cleavage site. This protease



**Figure 5** Asp2 β-secretase cleavage activity. **a**, Reverse phase HPLC profile showing the products of Asp2 cleavage. The amino-acid sequences of the parent peptide and the two hydrolysis products are indicated. **b**, Relative rates of hydrolysis of wild-type (triangles) and Swedish (squares) APP β-secretase peptides by Asp2.

contains an unusual C-terminal transmembrane domain that may help it colocalize with APP within cellular membranes where Aβ processing occurs. The expression pattern of Asp2 suggests a normal function in the brain, as well as in the exocrine pancreas. Our data indicate that Asp2 functions in the β-secretase pathway in cell lines of both somatic and neural origin, and that the enzyme meets many of the criteria expected of a APP β-secretase. Whether or not other enzymes also possess β-secretase activity is not excluded by these experiments. We note that identification of Asp2 as a candidate APP β-secretase has been reported independently by two other groups<sup>17,18</sup>. Thus, inhibitors of Asp2 will provide a new approach to the treatment of Alzheimer's disease. □

## Methods

### Reagents

Northern hybridization was performed using human tissue blots (Clontech). Chromosomal localization was performed by Genome Systems, Inc. *In situ* hybridization to human tissues was performed by LifeSpan Biosciences, Inc. We used 6E10 and 4G8 (Senetek), 22C11 (Boehringer-Mannheim), LN27 (Zymed Laboratories) Rb162 and Rb165 (New York Institute for Basic Research), 369 (Paul Greengard), and C8 (Dennis Selkoe) antibodies. Oligofectin-G and the Asp2-1 (5'-CCCTAATCACTGGCCGTTGGATGACT-3') and Asp2-2 (5'-GAATCATCTGTCACATGGCAAGCG-3') chimeric antisense oligomers were from Sequitur, Inc. APP constructs were assembled in the vector pIRES (Clontech). Asp2 constructs were assembled in the vector pcDNA3.1/hygro (Invitrogen). The sequence for the HEX-labelled TaqMan probe (Perkin Elmer) was 5'-AGGGCAA CAACGACGCGCAATTACA-3'. Amplification was performed with the primer pair 5'-TCAGAGCAGCAATGGCC-3' and 5'-GCCTGTAGTGGCTGGACA-3'.

### Transfection and Immunodetection

Oligofectin-G was used for lipid-mediated transfection of antisense oligomers into cultured cells according to the manufacturer's protocol. Supernatants and cell lysates were harvested 72 h after transfection. Transfection of plasmid DNA was performed using either the calcium phosphate method or Lipofectamine (GIBCO-BRL). The total amount of DNA used for transfection was held constant by adding empty vector DNA to the transfection mixture. Cell lysates and supernatants were harvested 48–160 h after transfection. Cells were lysed with 10 mM HEPES, pH 7.9, 150 mM NaCl, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 0.1 mM sodium vanadate and 1% NP-40. Equal amounts of protein (50 μg) were resolved on 4–12% Tricine gels (Novex), and transferred to nitrocellulose membranes for probing with 6E10, 22C11 or C8 antibodies. For immunoprecipitation, equal amounts of protein corresponding to one plate of cells were incubated with C8 or 369 antibody at 4 °C overnight, captured with protein A/protein G agarose beads, and processed for western blot detection of CTF99 with 6E10. The Aβ ELISA was done as described<sup>19</sup> using 6E10 monoclonal antibody as a capture antibody and biotinylated Rb162 or Rb165 antibody for detection of Aβ40 and Aβ42, respectively. The APPα ELISA used LN27 antibody as a capture antibody and biotinylated 6E10 for detection.

### Proteolytic activity assays

Recombinant Asp2 was purified from CHO cell membranes by solubilization in 25 mM Tris-HCl, pH 8.0, containing 50 mM β-octylglucoside followed by sequential chromatography on MonoQ and MonoS columns. Material prepared in this manner was more than 95% pure by SDS-PAGE analysis. Activity assays for Asp2 were performed using synthetic peptide substrates containing either the wild-type APP β-secretase site (SEVKM | DAEFR) or the Swedish KM → NL mutation (SEVNL | DAEFR). Reactions were performed in 50 mM 2-(N-morpholino)ethane-sulfonate, pH 5.5, containing 70 μM peptide substrate and recombinant Asp2 for various times at 37 °C. The reaction products were quantified by reverse phase HPLC. The sequence of both products was confirmed using Edman sequencing and MADLI-TOF mass spectrometry.

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## Purification and cloning of amyloid precursor protein beta-secretase from human brain

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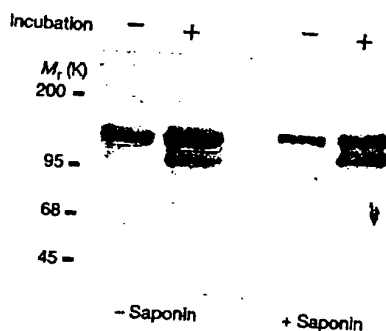
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Proteolytic processing of the amyloid precursor protein (APP) generates amyloid beta (Aβ) peptide, which is thought to be causal for the pathology and subsequent cognitive decline in Alzheimer's disease. Cleavage by beta-secretase at the amino terminus of the Aβ peptide sequence, between residues 671 and 672 of APP,

leads to the generation and extracellular release of beta-cleaved soluble APP<sup>1</sup>, and a corresponding cell-associated carboxy-terminal fragment. Cleavage of the C-terminal fragment by gamma-secretase(s) leads to the formation of Aβ. The pathogenic mutation K670M671 → N670L671 at the beta-secretase cleavage site in APP<sup>2</sup>, which was discovered in a Swedish family with familial Alzheimer's disease, leads to increased beta-secretase cleavage of the mutant substrate<sup>3</sup>. Here we describe a membrane-bound enzyme activity that cleaves full-length APP at the beta-secretase cleavage site, and find it to be the predominant beta-cleavage activity in human brain. We have purified this enzyme activity to homogeneity from human brain using a new substrate analogue inhibitor of the enzyme activity, and show that the purified enzyme has all the properties predicted for beta-secretase. Cloning and expression of the enzyme reveals that human brain beta-secretase is a new membrane-bound aspartic proteinase.

Beta-cleaved soluble APP (beta-sAPP) was detected in membranes isolated from 293 cells stably overexpressing the 'Swedish' mutation, SweAPP751, by western blot analyses using the beta-cleaved soluble APP (beta-sAPP)-specific antibody Sw192 (ref. 4). Incubation of the membranes at pH 5.5 led to an increase in the cell-associated beta-sAPP, and the appearance of a faster migrating species (Fig. 1). Treatment with O-glycanase resulted in the co-migration of both immunoreactive bands at the size of the lower band, which indicated that the smaller species resulted from beta-cleavage of membrane-associated N-glycosylated immature APP (data not shown). These results are consistent with the specific cleavage of the full-length APP at the beta-cleavage site by a membrane-bound proteinase activity. The membrane-bound beta-cleavage activity exhibited a preference for acidic pH, with an optimum value of pH 5.5. Co-incubation with class-specific protease inhibitors, such as pepstatin, E-64 or phenylmethylsulphonyl fluoride, did not affect the generation of the beta-cleaved APP (data not shown). Washing the membranes with 0.1% saponin under hypotonic conditions did not lead to loss of the membrane-associated beta-cleavage enzyme activity (Fig. 1); therefore, we extracted P2 membranes<sup>5</sup> in 0.1% Triton X-100, 0.1% Brij-35 or 0.1% beta-octylglucoside to test the solubility of enzyme activity. The soluble supernatant fractions were assayed for beta-cleavage activity, on an exogenous recombinant substrate, MBPC125Swe. Specific beta-cleavage was detected only in the Triton X-100 extracts.

We analysed various tissues and cell lines for beta-cleavage activity, by extracting P2 membranes from each source with 0.2% Triton X-100 and assaying for beta-cleavage (Fig. 2a). Human and mouse brain, and brain regions had uniformly high levels of enzyme activity, whereas little activity was detected in other tissues. In different cell lines, neurons had the highest level of enzyme activity, whereas 293, Cos and Chinese hamster ovary (CHO) cells had lower levels. Cells



**Figure 1** Endogenous substrate cleavage by beta-secretase in P2 membranes. Membranes were prepared from 293 cells stably transfected with APP751, and either extracted with 0.1% saponin (+ saponin) or used directly (- saponin). Membranes in 0.1 M sodium acetate, pH 5.5 and 2% DMSO were either incubated (+) or solubilized without incubation (-). Samples were analysed by immunoblotting with the beta-cleavage-specific 192sw antibody. M, relative molecular mass.

